

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

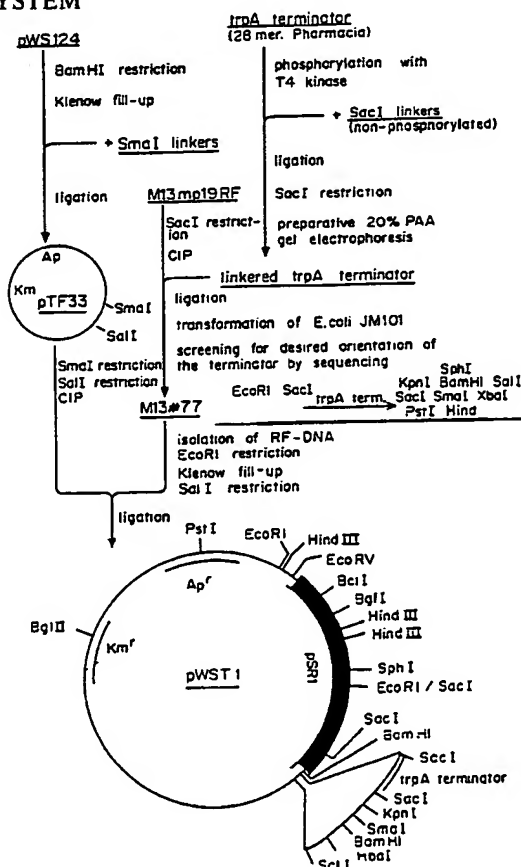
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification 4 : C12P 21/00, C12N 15/00, 1/20</p>	<p>A1</p>	<p>(11) International Publication Number: WO 88/ 09821</p> <p>(43) International Publication Date: 15 December 1988 (15.12.88)</p>
<p>(21) International Application Number: PCT/US88/01999</p> <p>(22) International Filing Date: 10 June 1988 (10.06.88)</p>		<p>(74) Agent: PABST, Patrea, L.; Kilpatrick &amp; Cody, 100 Peachtree Street, Suite 3100, Atlanta, GA 30043 (US).</p>
<p>(31) Priority Application Number: 061,883</p>		<p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</p>
<p>(32) Priority Date: 12 June 1987 (12.06.87)</p>		
<p>(33) Priority Country: US</p>		
<p>(71) Applicant: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).</p>		<p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(72) Inventors: LIEBL, Wolfgang ; Hart Str. 17, D-8057 Ech- ing (DE). SINSKEY, Anthony, J. ; 285 Common- wealth Avenue, Boston, MA 02115 (US).</p>		

**(54) Title: CORYNEFORM EXPRESSION AND SECRETION SYSTEM**

**(57) Abstract**

A coryneform expression and secretion system for homologous and heterologous genes consisting of the host, nucleotide sequences encoding a protein of interest, signals for expression and, optionally, targeting signals which direct membrane anchoring and for secretion and processing of the expressed protein. Regulatory signals may be utilized to control the rate and extent of expression and secretion. The system may further include compounds such as ionophores for altering the membrane transport of the host. The host itself may be mutated to alter transport, for example, by decreasing the mycolic acid content of *Corynebacterium* species. The preferred host is a *Corynebacterium* although other coryneforms deficient in extracellular protease production may also be used. *C. glutamicum* is used as a model organism for the secretion system. This Gram positive, non-pathogenic bacteria can efficiently utilize heterologous expression and secretion signals originating from a variety of both Gram negative and Gram positive bacteria to provide the basis for the overproduction and secretion of cloned gene products in a given *Corynebacterium* host, as demonstrated by the expression and secretion by *C. glutamicum* of a lipase encoded by a gene from *S. hyicus* and a thermonuclease encoded by a gene from *S. aureus*.



***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

-1-

CORYNEFORM EXPRESSION AND SECRETION SYSTEMBackground of the Invention

The present invention is in the area of improved procaryotic expression systems and, in particular, a Coryneform host system for the expression and excretion of gene products.

Coryneform bacteria are a taxonomically ill-defined group of Gram positive bacteria originally related by unique morphological features. These microorganisms occupy a wide variety of ecological niches and display an even broader array of interesting and useful properties. With the advent of systematic chemical analysis, there is considerable evidence indicating that the genus Corynebacterium is closely related to Mycobacterium and Norcardia. Included in the genus Corynebacterium are medically important species such as C. diphtheriae, animal pathogens such as C. renale, plant pathogens and diverse saprophytic, aerobic coryneform bacteria. The saprophytic coryneform bacteria are widely distributed in nature and include not only Corynebacterium species but also other bacteria including Arthrobacter, Brevibacterium, Cellulomonas, Microbacterium and Curtobacterium. The coryneform group thus represents an important source of enzymes, primary metabolites, and genetic material.

When cloning heterologous proteins for purification, it is often desirable to have the gene product hyperproduced and/or secreted by the host cells. The major advantages of secretion over

-2-

intracellular accumulation of recombinant proteins are an increase in yield and the facilitation of product purification. Translocation of proteins into or through membranes is an essential feature of prokaryotic and eukaryotic cells. Proteins that are partially or fully integrated into membranes, proteins that are associated or covalently bound to cell walls, or proteins that are secreted, must cross the cytoplasmic membrane.

Although initial investigations on protein export have been carried out with eukaryotic systems, there is an increasing interest in the mechanism and genetics of bacterial protein export. Benson et al., Cell 32, 1325-1335 (1985); D. Oliver, Ann.Rev.Microbiol. 39, 615-648 (1985); Randall and Hardy, Microbiol.Rev. 48, 290-298 (1984); and Pugsley and Schwartz, FEMS Microbiol.Rev. 48, 290-298 (1985), have recently reviewed this area. The Gram negative E. coli is the best-studied species among the prokaryotes. The most advanced experimental techniques have been tailored especially to fit the E. coli system. Despite the fact that the Gram positive cell wall has a simpler structure than its Gram negative counterpart, that Gram positive organisms are often very efficient in secreting proteins to the culture medium as compared with Gram negative organisms which normally cannot transport proteins beyond the outer membrane of their cell envelope, and that a vast number of extracellular proteins of Gram positive bacteria have been isolated and examined, including most bacterial

-3-

enzymes of commercial importance, the use of these organisms for basic investigations of protein export has been limited.

5 Most exported proteins, contrary to the majority of proteins localized in the cytoplasmic membrane, are synthesized as precursors with an N-terminal peptide extension (signal peptide) that is cleaved off in the course of translocation. Many  
10 of the bacterial and eukaryotic signal sequences that have been studied share striking structural similarities and are in fact interchangeable, as reported by several investigators. For example, the E. coli leader peptidase precisely recognizes and cleaves eukaryotic precursors.

15 Protein fusion experiments have demonstrated that a signal sequence alone is generally insufficient for the proper export of proteins. Several other types of targeting signals in addition to signal peptides have been identified. The most  
20 complex situation is found in eukaryotic cells where proteins must be directed to different subcellular compartments: endoplasmic reticulum, mitochondria, or chloroplasts. Additional information in the body of the mature protein may also be necessary. For  
25 example, posttranslational modification may contribute to the final localization of a protein, as seen with Gram negative lipoproteins and Gram positive lipopenicillinases.

Unfortunately, at this time, a good Gram  
30 positive cloning host has not been identified. The classic Gram positive cloning host, B. subtilis,

-4-

secretes extracellular proteases which attack heterologous proteins expressed in this organism, as reported by Ulmanen et al., J.Bacteriol. 162,176-182 (1985) and Doi et al., Trends in Biotech. 232-235 (1986). Consequently, there is a clear need for  
5 alternative Gram positive host organisms.

Protein secretion by coryneform bacteria has not been investigated, other than the secretion of diphtheria toxin by the pathogenic C. diptheriae upon  
10 infection with certain lysogenic tox<sup>+</sup> phages, reported by Pappenheimer, Ann.Rev.Biochem. 46,69-94 (1977) and Neville and Hudson, Ann.Rev.Biochem. 55,195-224 (1986). Even reports of the cloning in Corynebacterium hosts of the genes for two proteins  
15 which are normally exported in their native hosts, beta-lactamase from E. coli and alpha-amylase from Bacillus amyloliquefaciens, do not disclose whether or not these heterologous proteins were secreted.

It is therefore an object of the present  
20 invention to provide a Gram positive bacterial expression and secretion system.

It is another object of the present invention to characterize gene expression (replication, conjugal transfer and plasmid biology), in the Gram  
25 positive bacterial expression system.

It is yet another object of the present invention to further elucidate the genomic organization and structure of the Gram positive host, including the isolation and characterization of high efficiency and regulatable promoters.  
30

-5-

Summary of the Invention

A Coryneform expression and secretion system for cloned gene sequences consisting of the host nucleotide sequences encoding a protein of interest, signals for expression and, optionally, targeting signals which direct membrane anchoring and for secretion and processing of the expressed protein. In the preferred embodiment of the system for the expression and secretion of homologous and heterologous sequences, a Corynebacteria is selected as the host. The protein-encoding gene, transcriptional and translational start signals and a sequence coding for a secretion signal peptide are usually provided in a secretion vector, optionally including an inducible promoter. Regulatory signals may be utilized to control the rate and extent of expression and secretion. The system may further include compounds such as ionophores for altering the membrane transport of the host. The host itself may be mutated to alter transport, for example, by decreasing the mycolic acid content.

C. glutamicum is used as a model organism for the secretion system. This is a Gram positive, non-pathogenic bacterium which can efficiently utilize heterologous expression and secretion signals originating from a variety of both Gram negative and Gram positive bacteria to provide the basis for the overproduction and secretion of cloned gene products in a given Corynebacterium host.

The overexpression and secretion of foreign cloned genes in this system, and the exceptional



-6-

suitability of Corynebacterium as a host organism for the production and secretion of foreign gene products, is demonstrated by the expression and secretion by C. glutamicum of a lipase encoded by a gene from S. hyicus and a thermonuclease encoded by a gene from S. aureus.

#### Brief Description of the Drawings

Figure 1 is the construction of plasmid pWST1. A SmaI restriction site was introduced into the BamHI site of pWS124 on a synthetic oligonucleotide to create pTF33. The trpA terminator, from E.coli, was obtained as a 28 bp oligonucleotide and ligated to SacI linkers and cloned into M13 mp19 and screened by sequencing. Replicative form DNA of M13#77 was isolated and the terminator introduced into the SmaI-SacI region of pTF33 as a blunt ended EcoRI-SacI fragment to create pWST1.

Figure 2 is the restriction endonuclease map of pGN1. The C. glutamicum DNase gene was shown to reside on a 1.1 kb BamHI-BclI fragment within the 2.7 kb chromosomal DNA insert.

#### Detailed Description of the Invention

The present invention is a Coryneform expression and secretion system, and methods for its manufacture, demonstrated using C. glutamicum as a model organism for this group.

As discussed in the Background of the Invention, the coryneform group of bacteria encompasses a

-7-

number of diverse, Gram positive organisms, including Corynebacterium, Arthrobacteria, Nocardia, Mycobacterium, Cellulomonas, Microbacterium and Curto-  
5 bacterium. To be useful in the present invention, the bacterial host must be deficient in protease production, especially extracellular proteases. Preferably, the host should be amenable to large scale fermentation conditions, i.e., have simple  
10 nutritional requirements; grow within a wide range of temperatures, between 20 and 65°C; of food grade or being generally regarded as safe, such as Brevibacterium lactofermentum, Lactobaccillus species and Streptococci; non-spore forming, unlike most strains of Bacillus; transformable; and genetically stable,  
15 as opposed to many Bacillus and Streptomyces strain. Corynebacteria strains and particularly C. glutamicum, are preferred at this time.

In the past, it has not been possible to directly address questions of gene organization,  
20 structure and regulation of Corynebacterium at the molecular level due to deficiencies in the genetic tools that were available, even though Coryne-  
bacterium has long occupied a central role in the manufacture of a variety of primary metabolites  
25 including L-amino acids, nucleotides and organic acids by conventional fermentation. Several features of C. glutamicum make it especially desirable for extensive genetic studies: C. glutamicum is a nonpathogenic, food-grade microorganism, and the  
30 biochemistry and enzymology of C. glutamicum biosynthetic pathways have been extensively characterized.

-8-

As detailed below, there are a number of methods and variables to consider in the construction of an expression system using Corynebacterium as the host. Depending on the protein to expressed, the degree of regulation and quantity of expression desired, and whether or not the protein is to be secreted and/or targeted, the system can consist of as little as the host and an expression vector containing transcription and translation signals and the gene to be expressed. For the expressed protein to be secreted, the vector must further include targeting signals such as secretion signals, processing signals, and membrane anchoring signals. For control of the rate and extent of expression, the promotor may be placed under the control of a repressor or stimulatory protein. The expression/secretion system as a whole may also be modified by point mutations or deletions in any of the sequences discussed above, by the addition of membrane transport altering substances, or by mutations in the host affecting cell components such as the mycolic acid content of the cell wall.

The first consideration is to determine the protein to be expressed and to isolate a sequence encoding some or all of the protein. Methods for isolation of protein-encoding sequences are known to those skilled in the art of genetic engineering. Examples of the isolation and characterization of genes from C. glutamicum are provided.

The second consideration is the selection of an appropriate vector. A useful vector is pWST1 which

-9-

contains the E. coli trpA transcription terminator obtained from Pharmacia Fine Chemicals, Piscataway, New Jersey, immediately upstream of a polylinker cloning site. pWST1 is shown in Figure 1 and has particular utility in investigating promoter structure and function by eliminating read-through transcription from upstream promoters located within the cloning vector. Further examples of suitable vectors for use in Corynebacterium are described by U.S. Patent No. 4,649,119 to Sinskey et al. Other useful vectors, and methods for inserting the gene of interest into the vector, will be apparent to those skilled in the art.

Once the nucleotide sequence and the vector have been selected, sequences for the expression, regulation of expression, and post-translational characteristics of the expressed protein can be isolated and inserted into the vector. A number of promoters are useful in the present invention, including heterologous expression signals originating from a variety of both Gram negative and Gram positive bacteria including Escherichia, Bacillus, Staphylococcus and Streptococcus species. Of course, expression signals present in Corynebacterium are also useful. Indeed, an important feature of the present invention is not only the expression and secretion of gene products, but utilization of either homologous or heterologous gene sequences by the host.

An important element of the mechanism by which C. glutamicum mediates gene expression is through

-10-

promoter structure and the structure/function relationships. The promoters of naturally occurring genes can be determined by S1 nuclease mapping, described by Berk and Sharp in Cell, 12, 721(1977) using comparisons between known promoter sequences and the sequence which is being characterized. Identification of the transcription initiation sites and the alignment of upstream sequences can be used to identify consensus sequences. Deletion analysis of the isolated gene can also be used to confirm the promoter identification and to allow promoter replacement. In vitro generated deletions can be constructed using restriction enzymes or the exonuclease BAL31. The insertion of linkers facilitates subsequent cloning and sequencing. Restriction enzymes and linkers are commercially available from sources including Boehringer-Mannheim Biochemicals, Indianapolis, IN, and New England Biolabs Inc., Beverly, MA.

In some situations, it may be desirable to bring expression of a gene under control of a specific effector or repressor. In this case, either a regulatable promoter could be inserted prior to the gene, or the gene fused to the 3'end of a gene under the transcriptional control of a particular metabolite such as phenylalanine. Alternatively, an inducible promoter that allows product formation to be switched on during the appropriate growth phase, for example, as described Ghrayeb et al., EMBO J. 3,2437-2442 (1984), or mutant high copy vectors that allow overproduction of proteins could be used.

-11-

Secretion vectors are a specialized form of expression vectors and have to be specifically designed for each cloning system. They must carry efficient transcriptional and translational start signals as well as sequences coding for the N-terminal portion (at least the signal peptide) of a secreted protein. Secretion vectors have been constructed for various microbial cloning hosts, including E. coli, as reported by Takahara et al., J.Biol.Chem. 260, 2670-2674 (1985), B. subtilis, as reported by Palva et al., Proc.Natl.Acad.Sci.USA 79,5582-5586 (1982); Ohmura et al., J.Biochem. 95, 98-93 (1983); and Kovacevic et al., J.Bact. 162,521-528 (1985), staphylococci, as reported by Nilsson et al., Nucl. Acids Res. 13,1151-1162 (1985), and Liebl and Gotz, Mol.Gen.Genet. 204,166-173 (1986), and yeast, reported by Smith et al., Science 229,1219-1224 (1985).

A secretion signal sequence that functions in C. glutamicum can be selected by screening for expression of homologous or heterologous exoprotein genes in C. glutamicum and then used to form the basis of a secretion vector. The efficiency of this system can be tested by in-frame fusions of foreign genes, such as the structural gene for E. coli beta-lactamase devoid of its own expression/secretion signals, to the signal sequence. Expression of the gene fusion can be regulated either by the native promoter of the chosen exoprotein gene or by a homologous Corynebacterium promoter isolated in promoter search experiments. The amount and integrity of foreign protein released to the growth

-12-

medium upon transformation of C. glutamicum with the gene fusion demonstrates the capacity of this host organism to produce and secrete recombinant proteins.

5        Additionally, a sequence encoding an affinity tail, such as the IgG binding domain of protein A, could be inserted into the vector following the gene to be expressed to facilitate purification of the secreted product (Nilsson et al., Nucl. Acids Res.  
10    13, 1151-1162, (1985)).

      An element to be considered in protein secretion by C. glutamicum is the determination and characterization of targeting sequences which direct secretion membrane anchoring and post-translational  
15    processing of protein in C. glutamicum. One source of targeting signals are extracellular enzymes such as the lipase produced by P. acnes. Cloning of this lipase gene provides an exoprotein encoding gene from a species which is relatively closely related  
20    to C. glutamicum. Expression of the gene is detected by the presence of lipase activity. Staphylococci produce a number of extracellular proteins, some of which are bound to the cell wall of their native host. Examples of extracellular  
25    proteins produced by S. aureus are thermonuclease and protein A, both structurally and functionally well characterized proteins. The nucleotide sequences of the genes, as well as the N-terminal amino acid sequences of the mature proteins, are  
30    known. These genes can be placed under the transcriptional control of strong host promoters on an

-13-

expression vector for study, as well as placed under the control of their own native promoters. Analysis of the expression and localization of these proteins provides information on the recognition of  
5 heterologous targeting signals in coryneforms.

The energy requirement for secretion by C. glutamicum is another factor which can be characterized and altered as desired to modify secretion of proteins from the corynebacterium expression  
10 system. Transfer of proteins to the endoplasmic reticulum of eukaryotic cells is coupled to translation (cotranslational) and is driven by a mechanism that depends on the chain elongation process. Although some E. coli proteins (AMP C  
15 protein) exhibit complete cotranslational processing, others such as beta-lactamase and M13 code protein are synthesized to completion before export and processing are initiated. Most exported E. coli proteins show both cotranslational and post-transla-  
20 tional processing. Translocation and processing do not initiate until at least 80% of the polypeptide chain is complete. Protein export in E. coli depends on the total proton motive force (PMF), although the mechanism of the coupling membrane  
25 energy to transmembrane protein transport is unknown. The same coupling was found for the secretion of alpha-amylase in B. amyloliquefaciens. It is therefore expected that the same methods may be used to alter protein secretion in corynebacterium,  
30 in addition to modification by genetic engineering.

C. glutamicum clones expressing exoprotein genes can be used to investigate the energy



-14-

requirements of corynebacterium, and possible ways to alter the requirements and the effect of these alterations on protein expression and secretion. For example, the effect of varying the concentration of the ionophores carbonylcyanide M-chlorophenyl hydrazone (CCCP) and valinomycin in combination with K<sup>+</sup> on secretion can be determined. Inhibition of export and accumulation of cell-associated precursor molecules in the presence of these ionophores which reduce the PMF indicate the requirement of an energized membrane for translocation. One must take into consideration, however, that the effectiveness of these ionophores may be altered by the lipid-rich cell wall of the corynebacterium.

The mycolic acid layer of the corynebacterial cell wall may exert an effect on protein secretion. Production of mycolic acids (3-hydroxy-2-alkyl fatty acids) is a unique property of Corynebacterium, Mycobacterium, Nocardia and related taxa. The carbon chain link is taxon specific and is widely used in clarifying the taxonomy of the actinomycetes. Mycolic acids, arabinogalactan and peptidoglycan are interconnected and comprise the three main components of the cell walls of these bacteria. The function of the lipid outer cell wall layer in vivo is uncertain. It is likely that it plays a role in the interactions between the bacterium and its surroundings, for example, as a semipermeable membrane and in the selective uptake/release transport of compounds or compartmentation of certain proteins.

-15-

In order to both determine the effect of cell wall mycolic acids on protein secretion and to decrease the effect when it is so desired, mutants defective in mycolic acid synthesis are generated using mutation techniques known to those skilled in the art. It is preferred to generate mutants with reduced mycolic acid levels since totally deficient mutants are probably lethal. Deficient mutants can be screened for by staining with basic fuchsin in phenol water. Loss of the lipid rich outer coat decreases the retention of the stain.

The following examples demonstrate the isolation and characterization of native genes from C. glutamicum, the isolation and utilization of secretion signals for the export of the cloned gene products, and the overproduction of the cloned gene products in a Corynebacterium host.

Isolation and Identification of Corynebacterium Genes and Their Promoters

Genes encoding enzymes in various biosynthetic pathways, along with expression signals, can be isolated from a genomic library by complementation of an auxotrophic strains. Complementation analysis indicates that many C. glutamicum genes can be isolated by the heterologous complementation of E. coli auxotrophs followed by hybridization to elucidate the genomic organization of the isolated gene. Promoters for the gene of interest are then characterized. One technique is to use S1 nuclease mapping to identify the transcription start site of the gene. Comparison between known promoter sequences can be used to identify functionally impor-

-16-

tant nucleotides. Replacement of promoters can then be achieved using in vitro generated deletions.

5 The functional activity of the isolated gene and its promoters are tested by insertion of the recombinant plasmid into the expression host. Where a regulated gene is isolated, control of encoded activity by an effector proves that both the structural and regulatory sequences have been cloned. Northern hybridization analysis can be used to  
10 quantify the amount of gene specific message isolated from Corynebacteria grown in minimal medium with and without effector supplementation.

The mechanism of regulation of a particular system can be determined and then modified using one  
15 or more of the following methods. The involvement of a trans activity component in effector mediated repression is determined by cloning DNA fragments containing the proposed operator region, but not a functional gene, and introducing the cloned  
20 fragments into wild type C. glutamicum. Cloning of a larger portion of the operator region onto a multicopy plasmid could potentially influence transcription of the chromosomal encoded gene by titration of a repressor. The in vitro construction  
25 of point mutations and specific deletions which modify the operator region can also be used. Identification and modification of operator sequences could also enable in vitro isolation of repressor proteins for indirect, as well as direct,  
30 manipulation of expression.

-17-

Expression and Secretion of Non-Corynebacterium  
Genes in Corynebacterium

Although Staphylococci are not closely related to Corynebacterium, several Staphylococcus genes  
5 have been expressed in C. glutamicum. The lipase gene from Staphylococcus hyicus, described by Gotz et al., Nucl.Acids.Res. 13,5895-5906 (1985), and the thermonuclease gene from S. aureus, described by Shortle, Gene 22,181-189 (1983), were cloned into C.  
10 glutamicum.

Both the gene encoding nuclease production from S. aureus and the gene encoding lipase production from S. hyicus were readily expressed by C.  
glutamicum. The gene product was secreted into the  
15 culture medium. The cloned lipase gene is located downstream of the transcriptional terminator in the E. coli/C. glutamicum shuttle vector pWST1. It is presumed that in this situation the lipase gene is expressed via its native transcriptional promoter  
20 since the relative orientation of the gene in pWST1 does not influence its expression. C. glutamicum transformants harboring the lipase gene were analyzed by SDS polyacrylamide gel electrophoresis and an activity staining procedure (Gotz et al.,  
25 1985) which revealed a protein band at 45,000 Da with lipase activity.

Isolation and Utilization of Secretion Signals from  
Corynebacterium

Several proteins are released into the culture  
30 medium by the wild type strain of C. glutamicum. The gene coding for one such protein, DNase, was

-18-

cloned. Upon reintroduction into C. glutamicum, this gene results in the overproduction and secretion of active DNase. The cloned gene also provides a source of homologous expression and secretion signals useful in the construction of expression/secretion vectors for C. glutamicum.

The gene was isolated and utilized as follows. Chromosomal DNA from C. glutamicum was partially digested with MboI and 6 to 12 kb large fragments were ligated into the BamHI site of pHY416, described by Yoshihama et al., J.Bacteriol. 162,591-597 (1985), and Follettie and Sinskey, J.Bacteriol. 167,695-702 (1986). After transformation of the recombinant plasmids into C. glutamicum AS101 (his, Rif<sup>r</sup>), 1,500 Km<sup>r</sup> clones were screened on DNase agar plates (Oxoid USA Inc., Columbia, MD). Two clones showed increased DNase activity, both of which were found to contain the same 2.7 kb chromosomal DNA fragment in their respective plasmids. The restriction endonuclease map of one of these plasmids, designated pGN1, is shown in Figure 2. Deletion derivatives of pGN1 which were generated by BamHI or BclI digestion and religation (pGNBam4 and pGNBcl3, respectively) still conferred to C. glutamicum the DNase overproduction phenotype, indicating the location of the DNase gene in pGN1 to be on a 1.1 kb BamHI-BclI fragment. The nucleotide sequence of the 1.2 kb BamHI-EcoRI chromosomal DNA fragment carrying the corynebacterial DNase gene can be determined using techniques and equipment available to those skilled in the art.

-19-

This example appears to be the first case of the cloning and investigation of a homologous extracellular protein from C. glutamicum. It is useful in defining the structure of expression and secretion signals on the DNA and protein level and is therefore important for the future construction of secretion vectors for this cloning host.

Modifications and variations of the present invention, a coryneform expression system for cloned genes from Corynebacterium or foreign sources with the additional feature that the encoded protein is secreted from the host without undergoing extensive proteolytic degradation, will be obvious to those skilled in the art of genetic engineering from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

-20-

We Claim:

1. A bacterial expression and secretion system comprising:

5 a coryneform host, wherein said coryneform is deficient in the production of extracellular proteases, transformable and genetically stable.

2. The secretion system of claim 1 wherein said coryneform is also non-spore forming, food grade, has simple nutritional requirements, and grows at a temperature between 20°C and 65°C.

10 3. The secretion system of claim 1 comprising Corynebacterium.

4. The secretion system of claim 3 wherein said Corynebacterium is Corynebacterium glutamicum.

15 5. The secretion system of claim 3 further comprising cloned nucleotide sequences encoding expression signals.

6. The secretion system of claim 5 wherein said expression signals include a transcriptional and translational start sequences.

20 7. The secretion system of claim 5 wherein said expression signals include a termination sequence.

8. The secretion system of claim 5 wherein said expression signals are regulatable.

-21-

9. The secretion system of claim 5 wherein said expression signals are derived from non-Corynebacterium bacterial species.

5 10. The secretion system of claim 9 wherein said expression signals are derived from bacteria selected from the group consisting of Escherichia, Staphylococcus, Streptococcus and Bacillus.

10 11. The secretion system of claim 5 further comprising cloned nucleotide sequences encoding the protein to be secreted.

15 12. The secretion system of claim 5 comprising nucleotide sequences selected from the group consisting of sequences directing membrane anchoring, secretion, and processing of proteins in Corvne-  
bacterium.

13. The secretion system of claim 12 wherein said sequences are derived from a gene encoding an exoprotein.

20 14. The secretion system of claim 13 wherein said sequences are derived from a gene selected from the group consisting of the genes encoding lipase in P. acnes, lipase in Staphylococci, thermonuclease in S. aureus and protein A in S. aureus.



-22-

15. The secretion system of claim 13 wherein said sequences are isolated from the group consisting of Gram negative bacteria, Gram positive bacteria, yeast and eukaryotic cells.

5           16. The secretion system of claim 3 further comprising a compound altering the proton motive force of the Corynebacterium.

17. The secretion system of claim 16 wherein said compound is an ionophore.

10           18. The secretion system of claim 3 wherein the Corynebacterium is deficient in synthesis of mycolic acid.

15           19. The secretion system of claim 11 wherein said Corynebacterium is deficient in the production of the protein encoded by said nucleotide sequences.

20           20. A method for expressing and secreting cloned nucleotide sequences comprising providing a coryneform host deficient in the production of extracellular proteases, transformable, and genetically stable.

21. The method of claim 20 wherein said coryneform is also non-spore forming, food grade, has simple nutritional requirements, and grows at a temperature between 20°C and 65°C.

-23-

22. The method of claim 21 further comprising selecting the coryneform from the group consisting of Corynebacterium, Brevibacterium lactofermentum, Lactobacillus species and Streptococci.

5           23. The method of claim 20 further comprising providing the cloned nucleotide sequences to be expressed.

24. The method of claim 23 further comprising providing sequences for the expression of said  
10           cloned nucleotide sequences.

25. The method of claim 24 wherein the sequences for expression include transcription and translation start sequences.

26. The method of claim 25 wherein the se-  
15           quences for expression include a termination sequence.

27. The method of claim 24 further comprising providing sequences for the regulation of expression of said cloned nucleotide sequences.

20           28. The method of claim 20 further comprising providing nucleotide sequences selected from the group consisting of sequences directing membrane anchoring, secretion, and processing of protein.

-24-

29. The method of claim 28 wherein said coryneform is a Corynebacterium further comprising selecting said nucleotide sequences from the genes encoding lipase in P. acnes, lipase in  
5 Staphylococci, thermonuclease in S. aureus, and protein A in Staphylococci.

30. The method of claim 28 wherein said coryneform is a Corynebacterium further comprising selecting said sequences from sequences isolated  
10 from the group consisting of Gram negative bacteria, Gram positive bacteria, yeast and eukaryotic cells.

31. The method of claim 20 further comprising providing a compound altering the proton motive force of the coryneform.

15 32. The method of claim 31 wherein said compound is an ionophore.

33. The method of claim 31 further comprising selecting a Corynebacterium which is deficient in the synthesis of mycolic acid.

17/2

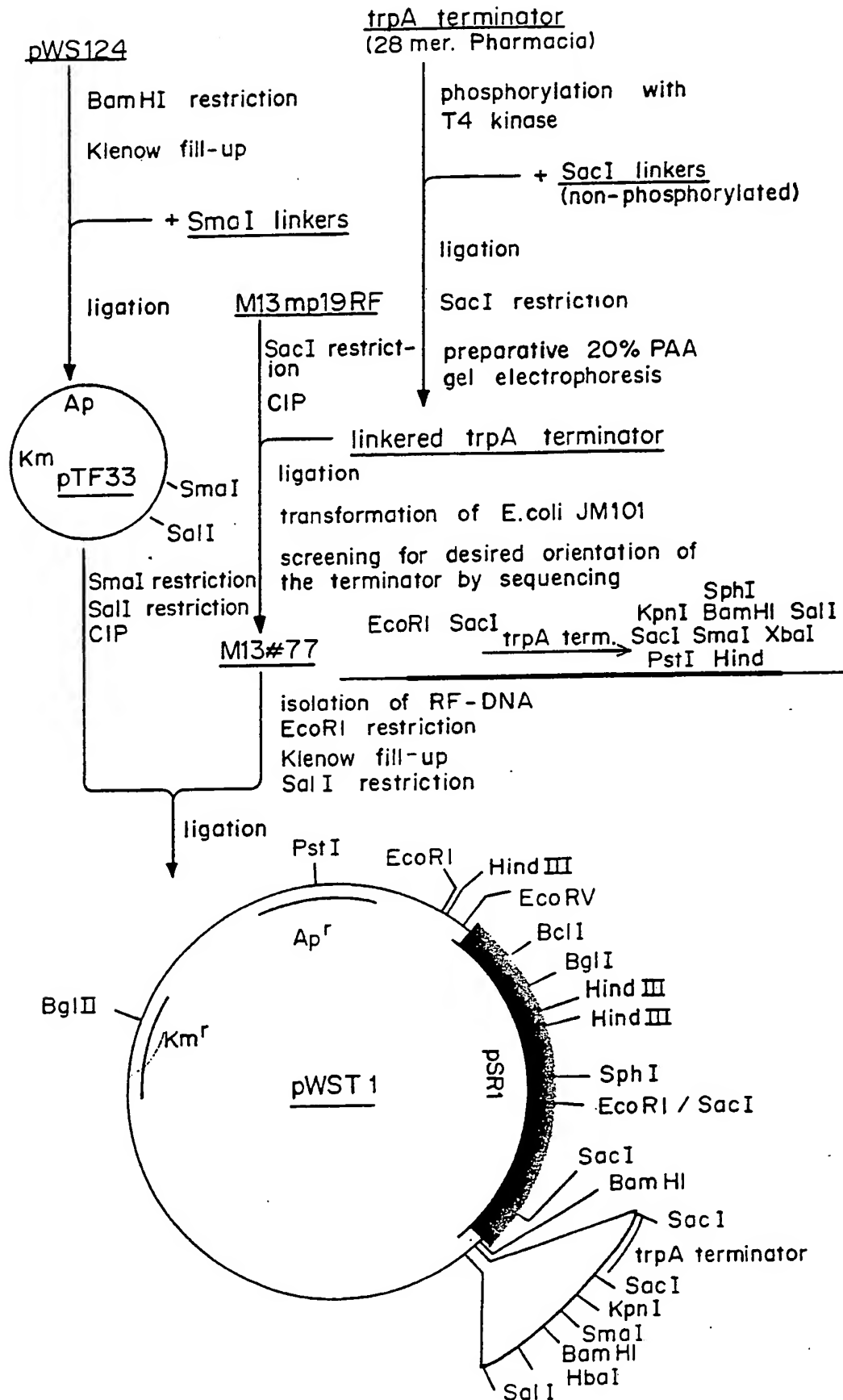


FIGURE 1

2/2

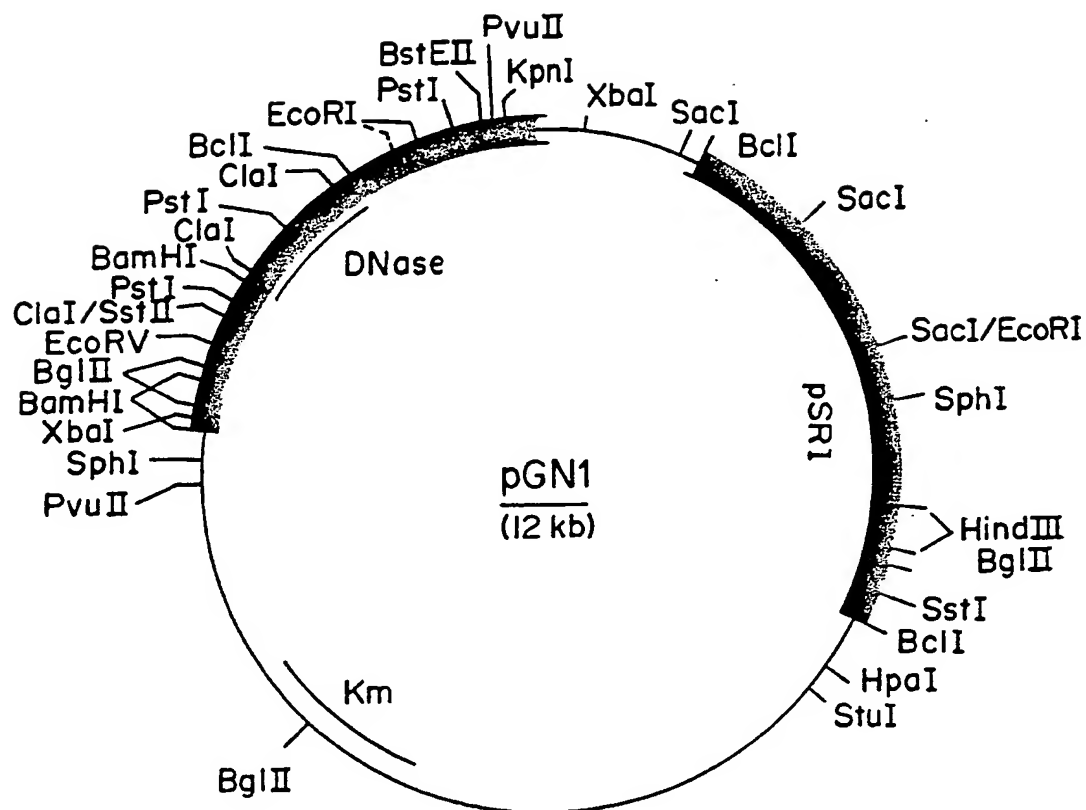


FIGURE 2

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/01999

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12P 21/00; C12N 15/00; C12N 1/20 U.S. 435/68; 435/172.3, 172.1, 435/253		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	435/68, 172.3, 253, 320, 172.1 935/29, 48	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched <sup>8</sup>		
Chemical Abstracts Data Base (CAS) 1967-1988. Biological Abstracts Data Base (BIOSIS) 1967-1988. Keywords: <u>Protease mutant; signal sequence, vector plasmid.</u>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y, E	US, A, 4,758,512 (GOLDBERG ET AL.) 19 July 1988 See entire document particularly columns 1, 2 and 6.	1-33
Y, P	US, A, 4,745,069 (MAYNE ET AL.) 17 May 1988 See entire document, particularly columns 1-7.	1-33
Y, P	US, A, 4,745,056 (GUTERMAN ET AL.) 17 May 1988 See entire document, particularly columns 1-3.	1-33
Y, P	US, A, 4,711,844 (CHANG) 8 December 1987, See entire document particularly columns 1-8.	1-33
Y, P	US, A, 4,711,843 (CHANG), 8 December 1987 See entire document particularly columns 3 and 4.	1-33
<div style="display: flex; justify-content: space-between;"> <div> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 06 September 1988		Date of Mailing of this International Search Report J 7 OCT 1988
International Searching Authority ISA/US		Signature of Authorized Officer THOMAS D. MAYS

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<u>Applied and Environmental Microbiology</u> , (Washington, D.C., U.S.A) Volume 53, issued February 1987 (FAHNESTOCK ET AL.), "Protease deficient <u>Bacillus subtilis</u> host strains for production of Staphylococcal protein A". See pages 379-384, particularly pages 374 and 375.	1-33
Y	<u>Journal of Bacteriology</u> (Washington, D.C. USA), Volume 167, issued August 1986 (FOLLETTIE ET AL.) "Molecular cloning and nucleotide sequence of the <u>Corynebacterium glutamicum pheA</u> gene". See pages 695-702, particularly pages 695 and 696.	1-33
Y	<u>Journal of Bacteriology</u> , (Washington, D.C. USA) Volume 162, issued May 1985 (YOSHIHAMA ET AL.) "Cloning vector system for <u>Corynebacterium glutamicum</u> ". See pages 591-597, particularly, 591-593.	1-33